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TITLE: Enhancing the Anti-tumor Activity of ErbB Blockers with Histone Deaccetylase(HDAC)Inhibition in Prostate Cancer Cell Lines

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14. ABSTRACT

PURPOSE: characterize the capacity of HDAC inhibitors to enhance the anti-tumor activity of anti-ErbB agents in prostate cancer cell lines. SCOPE: Interactions between these agents will be examined at both the cell signaling level, as well as through biologic end-points, including cellular proliferation, impact on cell cycle kinetics, invasion, and angiogenesis. MAJOR FINDINGS: HDAC inhibitors attenuate ErbB expression and the combination of HDAC inhibition and ErbB blockade resulted in near complete abrogation of EGFR and AKT signaling in the prostate cancer cell lines. HDAC inhibitors enhanced anti-proliferative effects and apoptosis induction of ErbB blockade in multiple cell lines. Preliminary gene expression profiles using cDNA arrays suggests multiple levels of potential synergy between ErbB and HDAC inhibitors. NEXT STEP: Continuing work with additional prostate cancer cell lines and examining other biologic end-points, including cell cycle kinetics, angiogenesis, and invasion. Promising results will then be evaluated in vivo.

15. SUBJECT TERMS
ErbB signal transduction; histone deacetylase (HDAC) inhibitors

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Introduction

The treatment of patients with locally advanced or recurrent prostate cancer remains a major challenge in oncology. Several novel targeted therapies, including anti-ErbB agents and HDAC (histone deacetylase) inhibitors show promise for clinical benefit in these patients. Despite high enthusiasm for these approaches, early clinical data with ErbB inhibitors, for example, suggests that only 10-20% patients respond favorably to treatment. Therefore, considerable effort is currently being focused toward increasing the anti-tumor activity of ErbB inhibitors by using them in combination with other agents. One particular class of agents with strong potential for enhancing the effects of ErbB inhibitors are HDAC inhibitors. HDAC inhibitors are a promising new class of anticancer agents which independently demonstrate activity in a wide variety of solid and hematologic tumors. During preliminary screening studies using cDNA microarrays, we identified the potential of HDAC inhibition to attenuate the transcription of ErbB family oncoproteins in human prostate cancer cell lines. Furthermore, our preliminary data suggests that when used cooperatively, HDAC inhibitors can enhance growth inhibition and apoptosis induction of ErbB receptor blockers in prostate cancer.

A potential relationship between HDAC inhibition and ErbB expression has only recently been reported and the mechanism responsible for this phenomenon has not been defined. Combining anti-ErbB blockers with HDAC inhibitors may hold strong promise for enhancing anti-tumor activity and overall clinical response. The primary objective of this grant proposal is to characterize the capacity of HDAC inhibitors to enhance the anti-tumor activity of anti-ErbB agents in prostate cancer cell lines. Interactions between these agents will be examined at both the cell signaling level, as well as through biologic end-points, including cellular proliferation, impact on cell cycle kinetics, invasion, and angiogenesis. Promising data would provide rationale for future clinical trial investigations.

Body

The initial tasks, as described in the approved Statement of Work, involve determining the capacity of HDAC inhibitors to abrogate ErbB expression and AKT activation. Our results are shown in Figures 1 and 2. As preliminary studies suggest,

- SAHA demonstrates the capacity of down-modulate both EGFR and ErbB2 expression in a panel of prostate cancer cell lines (Fig 1A/B) although did not seem to impact ErbB3/4 expression (Fig 1 C/D).
- Similarly, SAHA did not impact AKT expression, although did have the capacity to inhibit its activation in multiple prostate cancer cell lines (Fig 2 A/B).

Our next task was to conduct in vitro experimentation to characterize the capacity of HDAC inhibitors to enhance the effects of anti-ErbB agents in a panel of prostate cancer cell lines. Initial studies involved determining the impact of the single agent SAHA (HDAC inhibitor), Tarceva (EGFR inhibitor), and CI-1033 (pan-ErbB tyrosine kinase inhibitor) on cellular proliferation.

- The HDAC inhibitor SAHA demonstrated the capacity to inhibit cellular proliferation in a panel of prostate cancer cell lines (Fig. 3).

- Tarceva, on the other hand, had very little impact on cellular proliferation (Fig. 4).
- The pan-ErbB inhibitor CI-1033 demonstrated a significant impact on cellular proliferation (Fig. 5) in 2 of the 4 prostate cancer cell lines tested (DU145 and PC3).

The impact of HDAC inhibition on cellular proliferation was further evaluated by cell cycle analysis.

- The HDAC inhibitor SAHA demonstrated a dose dependant G2/M phase cell cycle arrest in the prostate cancer cell line DU145 (Fig. 6).

Initial studies examining the interaction between HDAC and ErbB inhibitors were performed at the level of cellular proliferation. Of the ErbB inhibitors, CI-1033 was chosen, as Tarceva demonstrated little activity in cell lines tested.

- The combination of CI-1033 and SAHA demonstrated an additive/synergistic impact on cellular proliferation in the DU145 and PC3 cell lines (Fig. 7).
- To determine if this interaction was additive or synergistic, serial concentrations of each agent was used to create an isobologram (Fig. 8) which demonstrated synergy with the combination of these agents in the DU145 cell line.

In addition to cellular proliferation, the interaction between HDAC and ErbB inhibitors was further evaluated at the level of apoptosis.

- The combination of the HDAC inhibitor SAHA and CI-1033 demonstrated a supra-additive induction of apoptosis, evaluated by caspase activity (Fig. 9A) and PARP cleavage (Fig. 9B).

The mechanism underlying these favorable interactions between HDAC and ErbB inhibitors was evaluated. Initial experiments involved the impact of HDAC inhibition on ErbB signaling.

- Both CI-1033 and SAHA demonstrated modest inhibition of EGFR and AKT activation. Combination of the two agents demonstrated a near complete abrogation of signaling (Fig. 10).

Since the initial summary statement, we are continued work examining other biologic end-points, including cell cycle kinetics, angiogenesis, and invasion as well as moved forth towards in vivo experimentation

Cell cycle kinetics

Cells were harvested after 48 hours exposure to SAHA, CI-1033, and, Tarceva, both independently and in combination. Cellular nuclei were stained using a propidium iodide solution and analyzed using flow cytometry. Resulting DNA distributions was analyzed for the proportion of cells in sub-G0, G1, S, and G2-M phases of the cell cycle.Previously described data (Fig. 6) demonstrated the capacity of SAHA to induce a G2/M dose dependent cell cycle arrest in the prostate cancer cell line DU145. Studies were then carried out in the MDA-PCa2b cell line. Further, the effect of EGFR inhibition, as the combination was evaluated. As demonstrated in Figure 11, SAHA had a similar G2/M phase cell cycle arrest in the MDA-PCa2b cell line. Alone, the EGFR inhibition resulted

in a G1 arrest in both cell lines, as would be expected. Interestingly, the combination of the two agents resulted in a cell cycle pattern similar to that of the HDAC inhibitor alone, resulting in no additive/synergistic impact on the S-phase fraction.

Apoptosis

Apoptosis was evaluated in the DU145 and MDA-PCa2b cell lines at sub-maximal doses of SAHA, CI-1033, and Tarceva, both independently and in combination. Quantitative analysis of apoptosis was obtained by evaluating caspase activity via fluorescence plate reader. As previously described (Fig. 9a), both SAHA and CI-1033 induced apoptosis, with the combination demonstrating a supra-additive effect. This was also validated by western blot analysis assessing PARP cleavage ratio (Fig. 9b). Further studies were performed in the MDA-PCa2b cell line. Although SAHA demonstrated a similar capacity to induce apoptosis, the MDA-PCA2b cell line was less responsive. Further, the combination of the two agents demonstrated no additive/synergistic interaction (Fig. 12). Tarceva was also tested both independently and in combination with SAHA. In these studies, the EGFR inhibitor had minimal influence on apoptosis in both cell lines, and the combination did not demonstrate any additivity/synergy.

Invasive Potential

The ability of tumor cells to spread and invade into basement membrane was determined using a system consisting of Matrigel on top of a polycarbonate filter using transwell inserts in a 12-well plate. Cells were be incubated for 24 hours in SAHA, CI-1033, and Tarceva, both independently and in combination. Cells that traversed through the Matrigel and polycarbonate filter will be fixed and stained using Diff-Quik fixative and staining solutions. The number of invasive cells were quantified by counting 9 random fields/sample under the microscope. As demonstrated in Figure 13, the HDAC inhibitor SAHA has a significant effect on invasive potential will this model. Neither Tarceva nor CI-1033 influenced invasion. The combination of the two agents did not enhance the interaction of SAHA alone on invasion.

Angiogenesis

The influence of SAHA, CI-1033, and Tarceva on angiogenesis, both independently and in combination, in the prostate cancer cell lines DU145 and MDA-PCa2b. Quantitative real time RT-PCR was used to determine levels of VEGF and HIF-1a transcript at 8 and 24 hours after exposure to SAHA, CI-1033, and Tarceva, both independently and in combination. All three agents demonstrated the capacity to inhibit VEGF and HIF-1a transcription. The various combinations were additive in all samples. No supra-additivity was noted (Fig. 14).

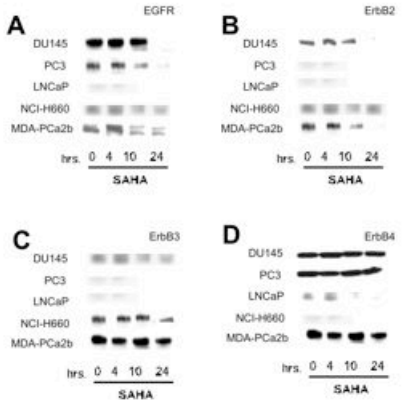
Endothelial cells have been shown to attach to reconstituted ECM matrix when plated, and are capable of forming tube-like structures. It is believed that these processes in ECM are representative of the early stages in angiogenesis during which vessels complete their differentiation and become stable. For reconstitution of basement membrane,

Matrigel was diluted two-fold with cold DMEM and added to the 24-well tissue culture plate at 4^{O}C. After gelation of Matrigel at 37^{O}C, HUVEC cells were added on top of the reconstructed basement membrane in the absence or presence of SAHA, CI-1033, and Tarceva both independently and in combination. Cells will be incubated for 6-18 hr to allow capillary-like structure formation. After solidification of agarose, the immobilized endotubes are fixed and stained. As noted in above experiments involving VEGF/HIF-1a transcription, both agents appeared to inhibit tube formation independently, although combination studies did not seem to enhance this response (Fig. 15).

In vivo response studies combining ErbB blockers with HDAC inhibition were perfomred in mouse xenograft models. After identifying sub-maximal concentrations of SAHA, CI-1033, and Tarceva was established, combination in vivo experimentation were performed. Mice were treated with daily doses of SAHA, CI-1033, and Tarceva, both independently and in combination, for 2-3 weeks. Tumor size was measured twice weekly during treatment and will be continued 2-3 weeks after treatment to evaluate for potential tumor re-growth. As demonstrated in Figure 16, combination studies demonstrated a supra-additive effect in the CI-1033/SAHA combination, although was additive/sub-additive inhibition of tumor growth when compared to single agent alone. Additionally cDNA microarray were performed on tumor specimens obtained from this in vivo model and results are currently being analyzed and validated by IHC to determine mechanisms underlying these interactions.

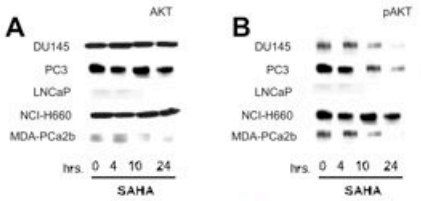
Supporting Data

Figure 1



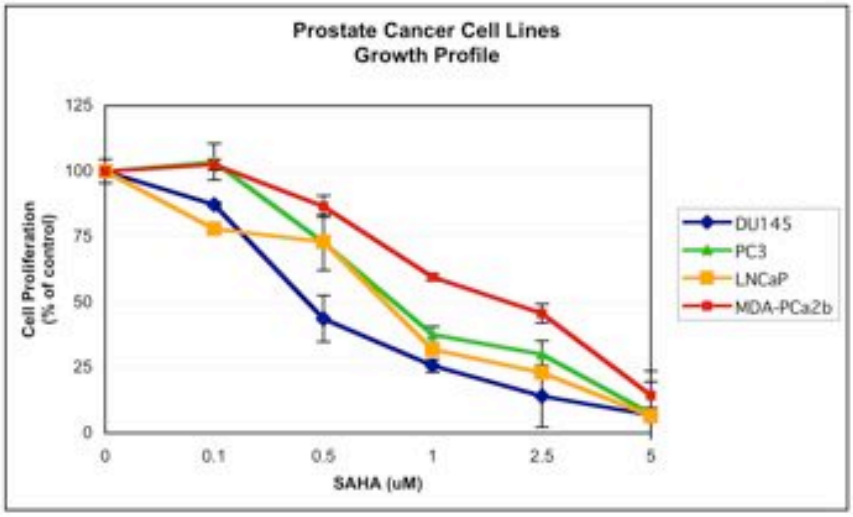
Western blot demonstrating the effect of SAHA on ErbB family member expression in prostate cancer cell lines 4, 10, and 24 hours post-treatment.

Figure 2



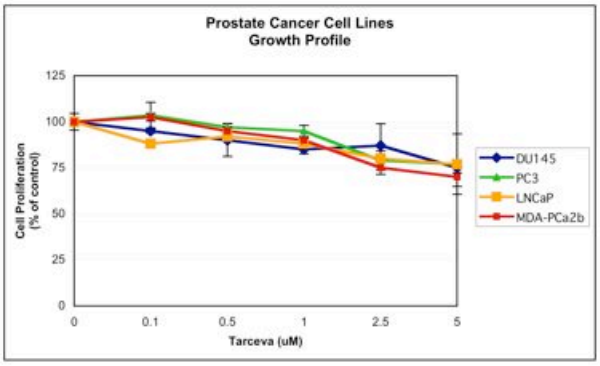
Western blot demonstrating the effect of SAHA on Akt expression and activity in prostate cancer cell lines 4, 10, and 24 hours post-treatment.

Figure 3



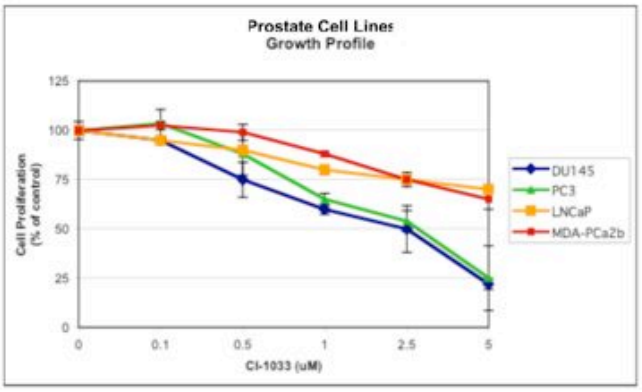
The effect of SAHA on cellular proliferation of prostate cancer cell lines in vitro.

Figure 4



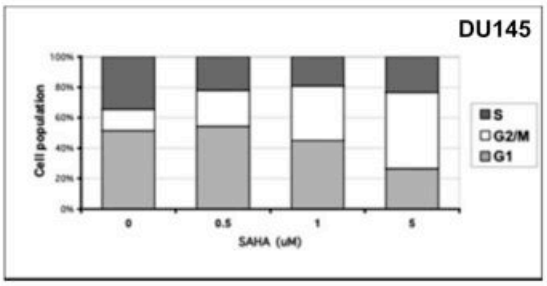
The effect of the EGFR inhibitir Tarceva on cellular proliferation of prostate cancer cell lines in vitro.

Figure 5



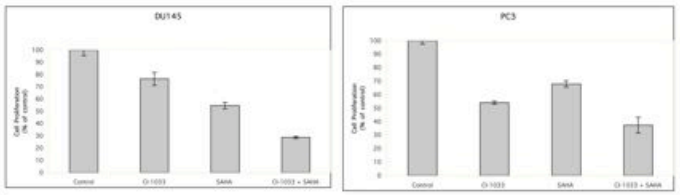
The effect of pan-ErbB tyrosine kinase inhibitor CI-1033 on cellular proliferation of prostate cancer cell lines in vitro.

Figure 6



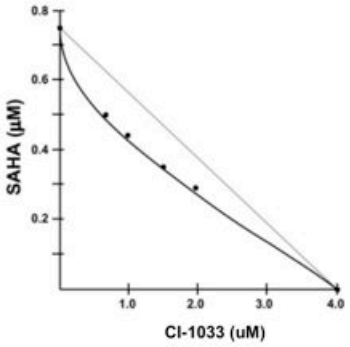
The effect of SAHA on cell cycle kinetics in the prostate cancer cell line DU145.

Figure 7



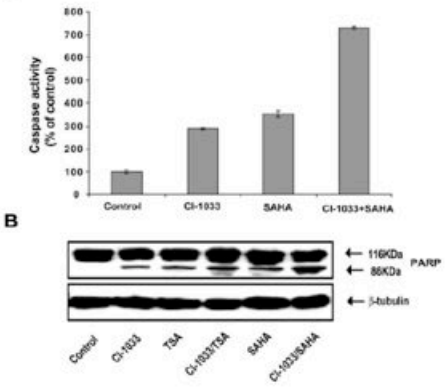
Combination studies demonstrating the interaction of the ErbB inhibitor (CI-1033) and HDAC inhibition (SAHA) in the prostate cancer cell lines DU145 and PC3.

Figure 8



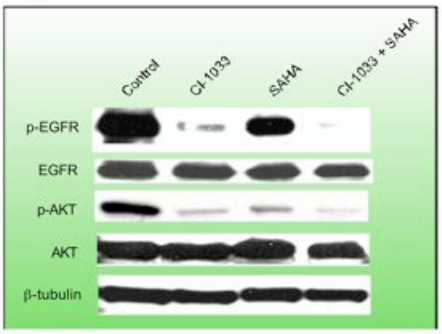
Isobologram demonstrating a supra-additive effect with the CI-1033 and SAHA combination in the prostate cancer cell line DU145.

Figure 9



The effect of SAHA and CI-1033 combination on apoptosis in prostate cancer cell line DU145. Apoptosis was evaluated using both a fluorescently labeled pan-caspase inhibitor (A) and western blot analysis evaluating cleaved PARP.

Figure 10



The effect of the CI-1033/SAHA combination on ErbB signaling in the prostate cancer cell line DU145.

Fig. 11

MDA	SAHA (uM)			
	0	**0.5**	**1.0**	**5.0**
G1	47%	49%	42%	22%
G2/M	13%	24%	55%	69%
S	40%	33%	3%	9%

MDA	Tarceva (uM)			
	0	**0.5**	**1.0**	**5.0**
G1	47%	56%	63%	73%
G2/M	13%	15%	19%	22%
S	40%	29%	18%	5%

DU145	Tarceva (uM)			
	0	**0.5**	**1.0**	**5.0**
G1	45%	58%	69%	80%
G2/M	17%	15%	19%	15%
S	38%	27%	12%	5%

MDA	SAHA /Tarceva (1.0 uM)			
	0	**0.5**	**1.0**	**5.0**
G1	63%	49%	42%	22%
G2/M	19%	24%	55%	69%
S	18%	33%	3%	9%

DU145	SAHA /Tarceva (1.0 uM)			
	0	**0.5**	**1.0**	**5.0**
G1	63%	47%	41%	20%
G2/M	19%	27%	58%	72%
S	18%	33%	2%	8%

Effect of SAHA, Tarceva, and the combination on cell cycle kinetics in prostate cancer cell lines.

Fig. 12

MDA	SAHA (uM)			
	0	**0.5**	**1.0**	**5.0**
Apoptosis	5%	9%	17%	34%
	CI-1033 (uM)			
	5%	6%	8%	12%
	Tarceva (uM)			
	4%	7%	4%	4%

	SAHA (1.0 uM) + CI-1033	**SAHA (0.5 uM) +Tarceva**
	19%	17%

Effect of SAHA, Tarceva, CI-1033 and the combination on apoptosis.

Fig. 13

DU145	SAHA (uM)			
	0	**0.5**	**1.0**	**5.0**
Invading colonies	23%	14%	9%	7%
	CI-1033 (uM)			
	23%	21%	17%	15%
	Tarceva (uM)			
	23%	24%	17%	22%

	SAHA (0.5 uM) + CI-1033	**SAHA (0.5 uM) + CI-1033**
Invading colonies	13%	14%

Effect of SAHA, Tarceva, CI-1033 and the combination on tumor invasion.

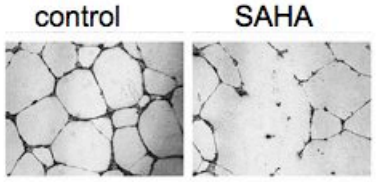
Fig. 14

DU145	VEGF (fold reduction)		Hif 1-a	
	8 Hr	24 Hr	8 Hr	24 Hr
SAHA	1.1	2.8	2.5	4.7
Tarceva	1.2	2.0	1.8	2.2
CI-1033	1.1	2.3	2.0	2.3
MDA	**8 Hr**	**24 Hr**	**8 Hr**	**24 Hr**
SAHA	1.1	2.8	2.5	4.7
Tarceva	1.2	2.0	1.8	2.2
CI-1033	1.1	2.3	2.0	2.3

DU145	VEGF	Hif 1-a
SAHA/Tarceva	3.2	6.2
SAHA/CI-1033	3.3	5.8
MDA		
SAHA/Tarceva	3.7	6.9
SAHA/CI-1033	2.9	5.3

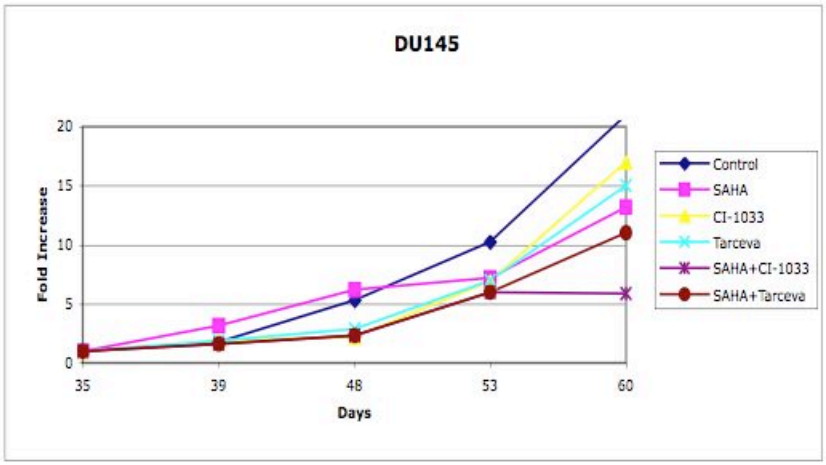
Effect of SAHA, Tarceva, CI-1033 and the combination on angiogenesis in prostate cancer cell lines

Fig. 15



Representation of the effect of HDAC inhibition on endothelial tube formation

Fig. 16



In vivo activity of SAHA, Tarceva, CI-1033, and the combination in the prostate cancer cell line DU145.

Key Research Accomplishments

1) HDAC inhibitors demonstrated the capacity to down-modulate ErbB expression, activity and down-stream signaling in prostate cancer cell lines.

2) The interaction between HDAC inhibitors and ErbB signaling was tested at multiple levels in prostate cancer cell lines. These studies suggest dual targeting of factors mediating angiogenesis to play a key role towards this favorable interaction.

3) In vitro studies were translated in vivo, demonstrating a favorable interaction with the combination. Array studies are currently being analyzed to provide further insight in mechanisms involved.

List of Reportable Outcomes

Chinnaiyan P, Varambally S, Tomlins SA, Ray S, Huang S, Chinnaiyan AM, Harari PM. Enhancing the antitumor activity of ErbB blockade with histone deacetylase (HDAC) inhibition. Int J Cancer. 2006 Feb 15;118(4):1041-50.

Conclusions

The results of the current study suggest the potential of HDAC inhibitors to enhance the antiproliferative and apoptotic effects induced by ErbB blockers in prostate cancer cell lines in vitro. Coadministration of these agents may represent a worthy strategy for more effective molecular targeting of the ErbB oncogenic pathway. Our findings demonstrate the capacity of HDAC inhibitors to down-modulate EGFR and ErbB2 expression in a variety of prostate cancer cell lines that moderately to significantly overexpress the ErbB oncoprotein. In addition, there appears to be a cooperative interaction involved in ErbB signaling, independent of protein expression. The dual targeting of factors mediating angiogenesis to play a key role towards this favorable interaction.

References

Chinnaiyan P, Varambally S, Tomlins SA, Ray S, Huang S, Chinnaiyan AM, Harari PM. Enhancing the antitumor activity of ErbB blockade with histone deacetylase (HDAC) inhibition. Int J Cancer. 2006 Feb 15;118(4):1041-50.

Personnel

Soma Ray (Research Assistant)